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ORIGINAL ARTICLE

Functional metagenomics reveals diverse β-lactamases in a remote Alaskan soil

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Despite the threat posed by antibiotic resistance in infectious bacteria, little is known about the diversity, distribution and origins of resistance genes, particularly among the as yet unculturable environmental bacteria. One potentially rich but largely unstudied environmental reservoir is soil. The complexity of its microbial community coupled with its high density of antibiotic-producing bacteria makes the soil a likely origin for diverse antibiotic resistance determinants. To investigate antibiotic resistance genes among uncultured bacteria in an undisturbed soil environment, we undertook a functional metagenomic analysis of a remote Alaskan soil. We report that this soil is a reservoir for β-lactamases that function in *Escherichia coli*, including divergent β-lactamases and the first bifunctional β-lactamase. Our findings suggest that even in the absence of selective pressure imposed by anthropogenic activity, the soil microbial community in an unpolluted site harbors unique and ancient β-lactam resistance determinants. Moreover, despite their evolutionary distance from previously known genes, the Alaskan β-lactamases confer resistance on *E. coli* without manipulating its gene expression machinery, demonstrating the potential for soil resistance genes to compromise human health, if transferred to pathogens.

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Introduction

The successful treatment of bacterial infections in humans is being thwarted by the prevalence of multiply antibiotic-resistant bacteria (Levy and O'Brien, 2005), thereby increasing disease incidence, longevity and mortality and the length and cost of hospital stays (Holmberg *et al.*, 1987; Cosgrove, 2006). To confront the resulting health crisis, we need a larger arsenal of antimicrobials (Levy and Marshall, 2004) and insight into antibiotic resistance, which will derive from understanding the ecology of resistance genes, including their origins, reservoirs and movement (Aminov and Mackie, 2007). Although antibiotic resistance has been studied intensively in clinical settings (Levy, 1997), little is known about the environmental reservoirs of resistance genes and their contribution to resistance in clinical settings. Identifying sources of resistance genes and tracking their movement from unmanaged ecosystems to the human milieu will advance the effort to combat antibiotic resistance in human pathogens. Owing to its complex microbial community, the soil is potentially a large environmental reservoir of resistance. A few studies that have addressed antibiotic resistance in the soil community (the soil resistome) have provided evidence that is consistent with the predicted diversity and abundance of resistance determinants (Riesenfeld *et al.*, 2004a; D'Costa *et al.*, 2006).

One group of resistance determinants predicted to be abundant in soil are β-lactamases. These enzymes hydrolyze the β-lactam class of antibiotics, such as penicillins and cephalosporins. The high efficacy and low toxicity (Livermore, 1996) of β-lactam antibiotics makes them among the most frequently prescribed antibiotics for humans and livestock, generating a powerful selection pressure for genes encoding resistance elements in environments proximal to human activity (Henriques *et al.*, 2006; Demaneche *et al.*, 2008). However, the empirical evidence is scarce regarding the origins of these genes (Hall and Barlow, 2004; Garau *et al.*, 2005) or their movement from unmanaged habitats to clinical settings. It seems likely that resistance genes are abundant in soil, even in the absence of anthropogenic selection pressure, because many soils may

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contain low concentrations of compounds that select for resistance. The soil is rich, for example, with microorganisms that produce β-lactam antibiotics, such as penicillins and cephalosporins (Martin and Liras, 1989).

Access to the wealth of as yet uncultured bacteria in soil is provided by the culture-independent method, metagenomics (Stein *et al.*, 1996; Riesenfeld *et al.*, 2004b), which involves extracting and cloning DNA directly from the environment. Analysis of metagenomic clones is often based on random sequencing (Venter *et al.*, 2004; Tringe *et al.*, 2005) or PCR amplification of target genes (de la Torre *et al.*, 2003; Henriques *et al.*, 2006; Demaneche *et al.*, 2008). Alternatively, functional metagenomics, which consists of heterologous expression of metagenomic DNA in a surrogate host and activity-based screening, provides the means to discover genes whose function might not be obvious from their sequence (Committee on Metagenomics: Challenges and Functional Applications, National Research Council, 2007).

This study was directed toward identifying genes that mediate resistance to β-lactam antibiotics in metagenomic libraries from a remote, Alaskan soil, which provides a comparative site for an ongoing study of antibiotic resistance in soils subjected to human intervention. The sampling site is an island in the large, fast-moving Tanana river in central Alaska. The site has no known exposure to antibiotics and is unlikely to have been visited by people other than researchers.

Materials and methods

Soil

Soil was collected from an island in the Tanana river in the National Science Foundation's Long-Term Ecological Research site at Bonanza Creek Experimental Forest near Fairbanks, Alaska, in July 2003, August 2004 and July 2005. All cores were taken from randomly chosen locations within the same plot. Soil cores were transported at 4 °C, stored at −20 °C and thawed at room temperature just before use. Owing to the periodic flooding of the river and deposition of glacial silt, the soil is visibly stratified into organic and mineral layers, which were treated as separate subsamples. The soil was aseptically removed from the core and either used directly (for extraction procedures requiring less than 1 g soil) or put through a sterile 5-mm sieve to remove roots and large particulates (for extraction procedures requiring more than 1 g soil).

Bacterial strains and culture conditions

Electrocompetent *Escherichia coli* TransforMax EPI300 cells (Epicentre, Madison, WI, USA) were used for library construction. All *E. coli* strains were grown in Luria–Bertani (LB) broth at 37 °C. When appropriate, media were amended with tetracycline at 20 μg ml^{-1}, chloramphenicol at 20 μg ml^{-1} or kanamycin at 20 μg ml^{-1} for plasmid maintenance.

Metagenomic library construction

Three methods were used for metagenomic library construction, as previously reported: bead-beating (libraries AK11 and AK12; FastDNA SPIN Kit (for soil), BIO101 systems, MP Biomedicals, Solon, OH, USA) (Riesenfeld *et al.*, 2004a), lysis within the soil matrix (libraries AK10, AK14, AK16, & AK18) (Williamson *et al.*, 2005) and cell separation (libraries AK20 and AK21; Liles *et al.*, 2008). The libraries were built in the following vectors: pCF430 (Newman and Fuqua, 1999), pCC1BAC (Epicentre) and pCC1FOS (CopyControl Fosmid Library Production Kit, Epicentre). Recombinant clones were scraped from selective agar into selective Luria–Bertani plus 20% glycerol and stored in pools at −80 °C.

Selection of resistant clones

All selections were carried out on LB agar with β-lactam antibiotics at the following concentrations: 50 μg ml^{-1} ampicillin (Research Products International Corp., Mount Prospect, IL, USA), 50 μg ml^{-1} carbenicillin (Fisher Scientific, Fair Lawn, NJ, USA), 16 μg ml^{-1} amoxicillin, 16 μg ml^{-1} cefamandole, 1 μg ml^{-1} ceftazidime, 50 μg ml^{-1} cephalexin, 100 μg ml^{-1} penicillin G and 12.5 μg ml^{-1} piperacillin (Sigma, St Louis, MO, USA). Metagenomic libraries were inoculated on the day of selection in 3 ml LB broth plus either tetracycline (libraries in pCF430) or chloramphenicol (libraries in pCC1BAC or pCC1FOS; Table 1). Cultures were incubated for 2–4 h at 37 °C with shaking. Cultures were plated at ~500 000 CFU per plate on LB agar plates containing the β-lactam antibiotics listed above. Half of the plates were incubated at 37 °C and half were incubated at 24 or 28 °C for up to 3 days. Resulting colonies were transferred onto LB agar plus either tetracycline or chloramphenicol and the appropriate β-lactam antibiotic, and incubated overnight. All resistant clones were evaluated by restriction digest and retransformation to confirm the phenotypes.

Antibiotic susceptibility testing

Minimum inhibitory concentration assays were performed according to CLSI (Clinical and Laboratory Standards Institute) guidelines NCCLS, 2004. Serial dilutions (512–0.5 μg ml^{-1}) of the β-lactam antibiotics listed above and cefoxitin (Sigma, St Louis, MO, USA) were made in Mueller–Hinton broth (Becton, Dickinson and Company, Sparks, MD, USA). Ten microliters containing $\sim 1 \times 10^5$ CFU of each of the clones listed in Table 3 were added to the appropriate wells. Minimum inhibitory concentration values are measured at twofold concentration increases. Assays were performed in duplicate and

Table 1 Alaskan soil metagenomic libraries used in this study[a]

Library	*AK Soil layer*[b]	*Vector*	*Clones*	*Avg insert size (Kb)*	*Bases (Mb)*
AK10	Organic	pCF430	16 300	10	173
AK11	Mineral	pCF430	36 800	5	184
AK12	Organic	pCF430	36 600	5	183
AK14	Organic	pCF430	105 800	5	529
AK16	Organic	pCF430	97 100	6	592
AK18	Organic	pCF430	34 500	10	338
AK20	Organic	pCC1BAC	48 100	8	385
AK21	Organic	pCC1FOS	337 000	30	10 100
Total			714 000		12 395

Abbreviations: AK, Alaskan; Avg, average.
[a]Libraries contain DNA extracted from soil and cloned into *E. coli* EPI300 cells (Epicentre Technologies, Madison, WI, USA), and were selected for resistance to eight β-lactam antibiotics.
[b]Organic-rich and mineral-rich layers were subsampled separately.

experiments were conducted at least twice with EPI300 *E. coli* carrying empty vector as the reference strain.

Identifying the active genes and sequencing active clones

Transposon mutagenesis was carried out *in vitro* according to the manufacturer's instructions with the GPS-1 Genome Priming System (New England Biolabs, Beverly, MA, USA). Insertion mutants that failed to grow on the appropriate β-lactam antibiotic(s) were identified as having insertions in the active gene and were sequenced using the manufacturer's primers. Additional random insertion mutants were chosen for sequencing the entire insert of βLR2, βLR3 and βLR17. βLR1 was sequenced by primer walking. These sequencing reactions were carried out at the University of Wisconsin-Madison DNA sequencing facility using Big Dye Terminator (v.3.1, Applied Biosystems, Foster City, CA, USA). Sequence reads were assembled using SeqMan (Lasergene software, DNASTAR, Madison, WI, USA). GeneQuest (DNASTAR) was used to identify putative open reading frames (ORFs), which were annotated using BLAST (Basic Local Alignment Search Tool) (Altschul *et al.*, 1990, 1997). As per the convention in the β-lactamase field, genes encoding β-lactamases were named '*bla*' (even though they do not all share a common ancestor), the protein products were named LRA# (β-lactam resistance from Alaskan soil), and the ORFs are designated $bla_{\mathrm{LRA-\#}}$.

The entire inserts of βLR5, βLR7, βLR8, βLR9, βLR10, βLR12, βLR13, βLR18 and βLR19 were sequenced by the US DOE Joint Genome Institute (Walnut Creek, CA, USA).

Subcloning bla_{LRA-13}

To determine whether both domains of $bla_{\mathrm{LRA-13}}$ were functional, the full-length ORF and individual domains were subcloned into pCF430 downstream of an arabinose-inducible promoter using preexisting *Nhe*I and *Hin*dIII sites. Appropriate restriction sites and a consensus *E. coli* Shine–Dalgarno site were engineered into the PCR primers (IDT, Coralville, IA, USA) upstream of the start codon. The full-length ORF subclone was designated pCFHBL01 (primers 5′-3′, F:cacggctagcaggaggatatta ATGAATTTTCGCCACATAGTCATGG, R:gcggaagctt CTAACGCTGGTCCAGTTGATCCAG; capital letters indicate coding region). The domain 1 (class D) subclone (pCFHBL02) comprised amino acids Met1–Leu252 (primers 5′-3′, F:cacggctagcaggaggatat taATGAATTTTCGCCACATAGTCATGG, R:gcggaag cttCTACAAGCCGGGGAGTTCCTTGAG), whereas the domain 2 (class C) subclone (pCFHBL03) comprised Met253–Arg609 (primers 5′-3′, F:cacggcta gcaggaggatattaATGATCAAGGATATGGTGGACCGC, R:gcggaagcttCTAACGCTGGTCCAGTTGATCCAG). The domain cutoff point was determined by BLAST (Altschul *et al.*, 1990, 1997) comparison and alignment with classes D and C β-lactamases, respectively; this comparison yielded a clear partition at the designated position.

Phylogenetic analyses

The top 100 homologs to each β-lactamase from Alaskan soil were identified using BLAST (Altschul *et al.*, 1990, 1997). The amino-acid sequences of all nonredundant β-lactamases were downloaded. Only those β-lactamases that had been studied in functional analyses were included in the phylogenetic analysis, thus excluding all homologs identified by sequence alone. For clarity of the figure, some β-lactamases from dense clades were eliminated before the final analysis to improve readability.

The amino-acid sequences for each class of β-lactamases (Ambler classes A, B, C and D were all treated separately) were aligned using ClustalX 1.83 (Thompson *et al.*, 1997) with the following parameters: pairwise gap opening penalty = 35, pairwise gap extension penalty = 0.75, multiple gap opening penalty = 15 and multiple gap extension penalty = 0.3. Alignments were optimized in GeneDoc (http://www.psc.edu/biomed/genedoc), and then subjected to neighbor-joining and maximum

parsimony analyses in Paup*4.0b10 (Swofford, 2003; 1000 bootstrap replicates) and in Bayesian analyses in MrBayes 3.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003; 200 000 Markov chain Monte Carlo (MCMC) generations, burnin of 200). ProTest (Abascal *et al.*, 2005) was used to determine that the fixed rate model, WAG + I + G (Whelan and Goldman, 2001; general reversible model of amino-acid replacement, fraction of sites invariant and γ distributed rates), best fit each data set under the Bayesian Information Criterion (BIC) framework. The model was incorporated into the Bayesian analysis of each set of β-lactamases with the following statements: 'lset rates = γ; prset aamodelpr = fixed(wag);'. Resulting trees were rooted with chromosomally encoded β-lactamases from the flavobacteria (classes A and B trees) and a Gram-positive organism (class C tree). The topology of each consensus parsimony tree conformed to the Bayesian trees except at nodes where no bootstrap value is reported, in which cases Bayesian trees provided better resolution (Figures 2a–c). Values are not reported for branches shorter than 0.01. Where the species on the tree are redundant, either the strain or β-lactamase name is reported.

Genbank accessions

The Alaskan soil metagenome project has been registered with the NCBI (National Center for Biotechnology Information) GenomeProject database (ID: 28853), and accession numbers are in Table 2.

Results

Identification of β-lactam antibiotic resistance genes from Alaskan soil metagenomic libraries

To capture β-lactam resistance elements from both culturable and as yet uncultured microorganisms, we constructed eight metagenomic libraries in *E. coli* containing a total of 12 Gigabases of DNA extracted from the soil (Table 1). Clones were selected from the libraries for their ability to grow in the presence of each of eight β-lactams. We identified 14 clones that contain metagenomic DNA that confers resistance on *E. coli* (Table 2). Most clones exhibited clinically relevant levels of resistance (NCCLS, 2004) to at least one of the eight β-lactam antibiotics tested (Table 3), including two clones that displayed resistance to β-lactams from both the penicillin and cephalosporin structural classes. The genes responsible for the resistance phenotypes were identified by transposon mutagenesis in 13 clones that harbored genes encoding β-lactamases (Table 2) representing new members of Ambler classes A, C, D (active site serine β-lactamases) and B (metallo-β-lactamases) (Ambler, 1980). The lone representative of the class D β-lactamases was linked with a class C β-lactamase as part of a single ORF harboring two full-length genes; this is the first report of a bifunctional β-lactamase.

Identification and characterization of the bifunctional β-lactamase

The bifunctional β-lactamase was encoded by a clone carrying a 42-kb metagenomic DNA insert (βLR13, Table 3). A single ORF, designated bla_{LRA-13}, was responsible for resistance (Figure 1a and Table 3), and its deduced protein contains 609 amino acids, which is nearly twice the size of all previously reported β-lactamases. The C-terminus (356 amino acids) aligns with class C β-lactamases (Table 2, Figure 1a) and the N-terminus (253 amino acids) aligns with class D β-lactamases (Table 2, Figure 1a). Therefore, bla_{LRA-13} appears to be a natural fusion between two full-length enzymes. Neither domain has significant deletions or insertions, and βLR13 shows even G + C content through-

Table 2 Clones from Alaskan soil metagenomic libraries that confer resistance to β-lactam antibiotics on *Escherichia coli*

Clone name	*GenBank accession number*[a]	*Active gene*[b]	*Resistance gene (% amino-acid identity/% similarity, homolog)*
βLR1	EU408346	bla_{LRA-1}	67/80, class A β-lactamase from *Burkholderia cepacia*
βLR2	EU408347	bla_{LRA-2}	45/62, class B β-lactamase from *Janthinobacterium lividum*
βLR3	EU408348	bla_{LRA-3}	48/57, class B β-lactamase from *Chryseobacterium meningosepticum*
βLR5	EU408358	bla_{LRA-5}	35/54, probable class A β-lactamase from *Gloeobacter violaceus*
βLR7	EU408356	bla_{LRA-7}	42/57, class B β-lactamase from *Elizabethkingia meningoseptica*
βLR8	EU408349	bla_{LRA-8}	45/58, class B β-lactamase from *Janthinobacterium lividum*
βLR9	EU408350	bla_{LRA-9}	36/54, class B β-lactamase from *Elizabethkingia meningoseptica*
βLR10	EU408357	bla_{LRA-10}	57/74, class C β-lactamase from *Mycobacterium smegmatis* MC2 155
βLR12	EU408351	bla_{LRA-12}	61/75, class B β-lactamase from *Chryseobacterium meningosepticum*
βLR13	EU408352	bla_{LRA-13}	1. 54/72, class C β-lactamase from *Shewanella baltica* OS195 2. 56/72, class D β-lactamase from *Burkholderia thailandensis* E264
βLR17	EU408354	bla_{LRA-17}	51/65, class B β-lactamase from *Chryseobacterium meningosepticum*
βLR18	EU408355	bla_{LRA-18}	54/73, class C β-lactamase from *Acinetobacter baylyi*
βLR19	EU408359	bla_{LRA-19}	59/71, class B β-lactamase from *Chryseobacterium meningosepticum*

[a]Full-length DNA sequences of clones were deposited.
[b]Open reading frames encoding β-lactamases were all designated '$bla_{LRA-\#}$'. This notation follows the convention of the β-lactamase field.

Table 3 MIC values (μg ml^{-1}) of clones and three subclones from Alaskan soil metagenomic libraries against eight β-lactam antibiotics

	Amoxicillin	*Ampicillin*	*Carbenicillin*	*Piperacillin*	*Cephalexin*	*Cefamandole*	*Ceftazidime*	*Cefoxitin*
pCF430[a]	8	8	16	4	8	1	0.5	4
βLR1	128	128	512	16	16	8	1	4
βLR2	64	128	512	8	32	8	32	8
βLR3	8	16	8	8	8	2	8	8
βLR16	8	16	128	4	8	0.5	1	ND
pCFHBL01[b]	32	64	128	128	16	0.5	0.5	4
pCFHBL02[b]	32	64	64	256	8	0.5	0.5	4
pCFHBL03[b]	4	8	16	512	128	1	4	4
pCC1FOS[a]	4	8	8	4	8	0.5	0.5	2
βLR5	8	16	32	8	32	4	32	16
βLR7	8	16	32	4	16	8	128	8
βLR8	>512	>512	>512	>512	64	128	128	32
βLR9	8	8	16	4	8	4	32	4
βLR10	16	64	32	4	32	4	2	4
βLR12	128	256	512	256	32	4	16	16
βLR13	64	64	64	64	64	1	2	8
βLR17	16	8	8	8	16	8	8	32
βLR18	64	64	16	8	>512	16	2	32
βLR19	256	32	>512	64	32	4	4	16

Abbreviations: MIC, minimum inhibitory concentration; ND, not determined.
[a]MIC values of empty cloning vector in metagenomic host cells (*Escherichia coli* Epi300). Clones are grouped by vector.
[b]Subclones of bla_{LRA-13} (see Figure 1 for a visual representation).

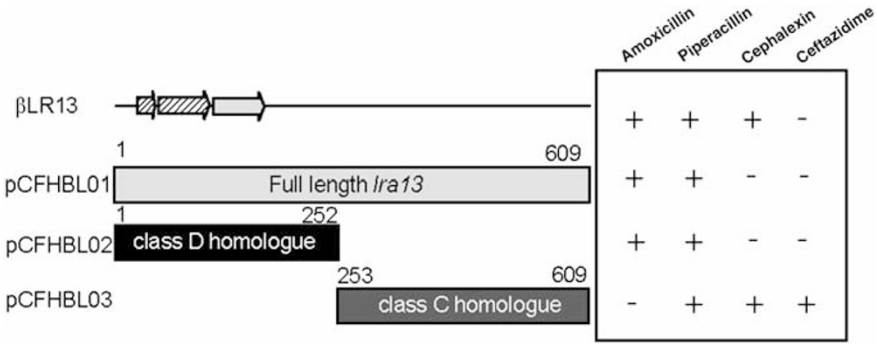


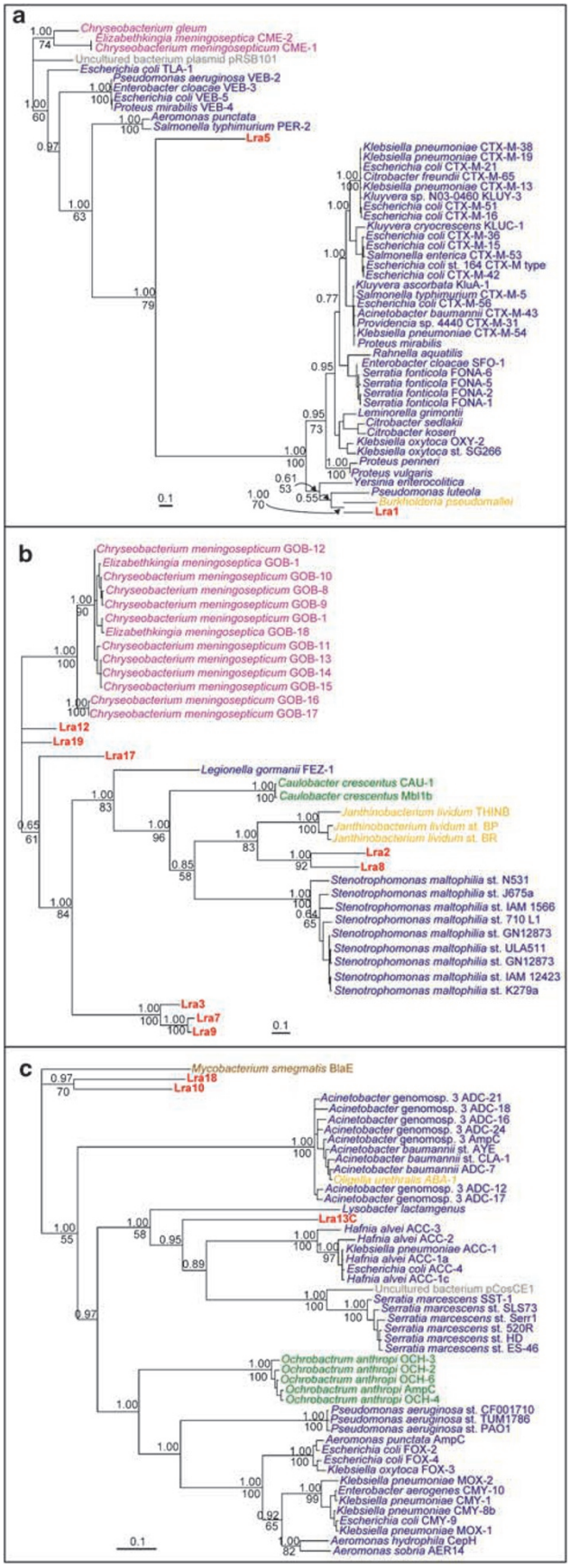
Figure 1 Resistance phenotypes of βLR13, which is a clone containing a 42-Kb insert carrying the gene encoding the bifunctional β-lactamase LRA-13, and subclones. Hatched arrows represent putative regulatory elements upstream bla_{LRA-13}, which is represented by a light gray arrow. Numbers above the rectangles represent amino-acid residues. Resistance phenotypes were designated '+' if the *E. coli* clone showed greater than twofold increase in resistance over the negative control in MIC assays, which were performed in duplicate at least twice. *E. coli*, *Escherichia coli;* MIC, minimum inhibitory concentration.

out the bla_{LRA-13} ORF and as well as in the flanking DNA (data not shown).

To determine whether both domains are functional, the full-length ORF and individual domains of the bifunctional β-lactamase were subcloned and tested for resistance (Figure 1a). The subclone containing the full-length ORF exhibits the levels of resistance similar to the parent strain in assays that measure minimum inhibitory concentration (Table 3 and Figure 1a), with minor differences likely attributable to the differences in gene expression levels and experimental variation. Each of the two β-lactamase domains contributes to the resistance profile of the full-length ORF, demonstrating that the hybrid β-lactamase is indeed a bifunctional enzyme (Figure 1a). Resistance to amoxicillin, ampicillin and carbenicillin is almost exclusively imparted by the N-terminal (class D) domain of the hybrid enzyme, and resistance to cephalexin is because of the C-terminal (class C) domain (Figure 1a). In general, expression of the C-terminal domain confers resistance to cephalosporin-type β-lactams; this includes β-lactams to which the full-length clone confers little to no resistance. Intriguingly, there is an overlap in specificity with respect to piperacillin resistance. The fusion, therefore, expands the substrate specificity beyond what either domain could hydrolyze alone.

Phylogenetic and functional analyses of Alaskan soil β-lactamases

The amino-acid sequences of class A β-lactamases from Alaskan soil, LRA-1 and LRA-5, are highly divergent from each other (Figure 2a). LRA-1 clusters with the chromosomally encoded β-lactamases from



Burkholderia pseudomallei, Pseudomonas luteola and *Yersinia entercolitica*, all of which are distantly related to the clinically relevant CTX-M family of β-lactamases (Figure 2a). In contrast, LRA-5 is homologous to the predicted genes encoding putative β-lactamases found in sequenced genomes (Table 2 and data not shown), and has low similarity to functionally characterized β-lactamases (Figure 2a). Indeed, the length of the branch to LRA-5 indicates that it is a distant relative of both characterized β-lactamases and their ancestors (Figure 2a). In addition to their sequence divergence, LRA-1 and LRA-5 confer different functional profiles; the metagenomic *E. coli* clone containing LRA-1 is broadly resistant to the members of the penicillin structural class, whereas the clone containing LRA-5 is resistant to the members of the cephalosporin structural class (Table 3).

All of the recovered class B β-lactamases fall into one (B3) of the three subgroups (Rasmussen and Bush, 1997) of known metallo-β-lactamases (Figure 2b). The length of the branches leading to the Alaskan soil metallo-β-lactamases from the nearest node indicate that they are more closely related to ancestral enzymes than any previously characterized enzyme to that same ancestor. All but two of the Alaskan soil metallo-β-lactamases diverge deeply from each other and others in subgroup B3. Interestingly, the relatedness of the Alaskan soil metallo-β-lactamases does not necessarily correspond to the similarity in functional profiles. For example, although LRA-2 and LRA-8 cluster together phylogenetically (Figure 2b), the clone containing LRA-8 confers resistance to *E. coli* to a broader spectrum of antibiotics than does the clone containing LRA-2. In contrast, two closely related β-lactamases, LRA-7 and LRA-9, show similar resistance profiles (within the twofold concentration changes) on all antibiotics tested (Table 3).

The Alaskan soil class C β-lactamases, LRA-10 and LRA-18, cluster with BlaE from *Mycobacterium smegmatis*, which is the only class C β-lactamase from a Gram-positive organism to which LRA-10 and LRA-18 aligned (Figure 2c). These sequences form a distinct clade that is apart from the clades of class C β-lactamases of Gram-negative organisms. The class C domain of LRA-13 does not cluster with the other class C β-lactamases from Alaskan soil (Figure 2c). All of the class C β-lactamases from Alaskan soil have similar functional profiles, except

Figure 2 Bayesian evolutionary trees of aligned amino-acid sequences of (**a**) class A β-lactamases, (**b**) class B metallo-β-lactamases and (**c**) class C β-lactamases. Bayesian posterior probabilities are shown above the nodes, and bootstrap values based on parsimony analysis are below the nodes. β-lactamases from Alaskan metagenomic libraries are shown in red. β-lactamases from other bacteria are color coded at the class level as follows: actinobacteria, brown; flavobacteria, purple; α-proteobacteria, green; β-proteobacteria, gold; γ-proteobacteria, blue; uncultured plasmid, gray. See Supplementary Table 1 for amino-acid sequence accession numbers. Scale bar = 0.1 changes/site.

that the clone containing LRA-18 (βLR18) confers greater resistance on *E. coli* to cephalosporins than do the other class C-containing clones (Table 3).

Evolutionary trees with the class D β-lactamase domain of the bifunctional β-lactamase were poorly supported in both Bayesian and parsimony analyses, and therefore an analysis of this class was not possible (data not shown).

Discussion

Functional metagenomic analysis of Alaskan soil revealed a gene encoding a bifunctional β-lactamase, $bla_{\mathrm{LRA-13}}$. Although bifunctional enzymes are rare in bacteria (Kim *et al.*, 2007), four bifunctional aminoglycoside resistance genes have been reported previously (Vakulenko and Mobashery, 2003; Kim *et al.*, 2007). In each of these, as in $bla_{\mathrm{LRA-13}}$, the fusion confers expanded substrate specificity compared with either domain alone, suggesting that pathogenic organisms harboring these genes might have a selective advantage in a clinical environment. This advantage may be because of tighter regulation of expression, greater efficiency and convenience for mobilizability of a gene encoding a bifunctional enzyme than two individual resistance genes. Indeed, the four aminoglycoside resistance gene fusion events are shown to be recent and because of an extensive selective pressure (Kim *et al.*, 2007). This is in contrast to the bifunctional β-lactamase from Alaskan soil, whose domains are distantly related to known β-lactamases, and therefore the fusion is not likely to have arisen because of the selective pressure from modern use of antibiotics.

We also found diverse and ancient β-lactamases in a soil environment with minimal human-induced selective pressure. We identified β-lactamases from each of the four structural classes of β-lactamases. Evolutionary analyses of the deduced amino-acid sequences of the classes A, B and C β-lactamases showed that the Alaskan β-lactamases diverge deeply from previously described β-lactamases. This supports our hypothesis that the Alaskan soil β-lactamases are more closely related to the ancestral β-lactamases than β-lactamases isolated in clinical settings because of the unpolluted nature of the sampling site, which contrasts with the intensely selective environment of the clinic. Additionally, the diversity of the Alaskan soil β-lactamases is exemplified functionally, with no two β-lactamases having identical phenotypes, and many β-lactamases conferring high levels (in clinical treatment terms) of resistance on *E. coli*. Taken together, our results indicate that β-lactamases from Alaskan soil are diverse, are more closely related to ancestral homologs than those isolated in clinical settings and are capable of conferring resistance on *E. coli* despite this evolutionary distance.

More metallo-β-lactamases than any other class were isolated from Alaskan soil, and all of them belong to one (B3) of three subgroups of metallo-β-lactamases (Rasmussen and Bush, 1997). Homologs of enzymes in subgroup B3 are found in both bacteria and archaea, and the β-lactamase function of B3 enzymes is thought to have evolved more than 2.2 billion years ago (Hall *et al.*, 2004). In addition to their primordial existence, metallo-β-lactamases belong to the zinc metallo-hydrolase family, of which there are more than 6000 members that catalyze a broad range of reactions in all domains of life (Bebrone, 2007). These data suggest that the presence of metallo-β-lactamases in an environment that is not highly selective, such as Alaskan soil, could be a result of divergent evolution from non-β-lactam hydrolyzing members of the zinc metallo-hydrolase family. Perhaps the metallo-β-lactamases are more common because of the wide range of reactions carried out by the members of zinc metallo-hydrolase family (Daiyasu *et al.*, 2001). In this case, diverse substrates, in addition to β-lactams, might select for genes that encode metallo-β-lactamases.

The function of any of the β-lactamases in the native soil microbial community is not known, although the results of this study stimulate intriguing hypotheses. Certain soil microorganisms are known to produce β-lactams, and although the antibiotics are not present at therapeutic levels, there may be sufficient concentrations to select for resistance in subpopulations within a community. Moreover, antibiotics themselves are only one of the many environmental factors that may affect the frequency of resistance genes (Singer *et al.*, 2006). Antibiotic resistance genes may play roles outside of the 'war' metaphor, which is the traditional paradigm for antibiotics and resistance genes. Subminimal inhibitory concentrations of antibiotics have been demonstrated to modulate bacterial gene expression, and, consequently, Davies *et al.* (2006) has proposed that antibiotics evolved as signaling molecules rather than weapons . In this scenario, β-lactamases in nature might disrupt such signaling (Yim *et al.*, 2007), much like enzymes shown to hydrolyze the acylhomoserine lactone signaling molecules that are ubiquitous among the proteobacteria (Dong and Zhang, 2005).

As microbial antibiotic resistance continues to gain traction in clinical settings, it is imperative that we extend these studies to other environments and antibiotics to understand the ecology of resistance genes in infectious disease and in natural microbial communities. Resistance genes residing in the environmental reservoir do pose a threat to human health, if they migrate to clinical settings and transfer to pathogens, in which they could be expressed as demonstrated here.

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